

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
7 December 2000 (07.12.2000)

PCT

(10) International Publication Number
WO 00/72851 A1

(51) International Patent Classification⁷: **A61K 31/74**

(21) International Application Number: PCT/US00/14353

(22) International Filing Date: 24 May 2000 (24.05.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/322,903 1 June 1999 (01.06.1999) US
09/406,972 28 September 1999 (28.09.1999) US
09/447,456 22 November 1999 (22.11.1999) US
09/494,814 31 January 2000 (31.01.2000) US

(71) Applicant (for all designated States except US): **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 12th floor, 1111 Franklin Street, Oakland, CA 94607-5200 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **PRUSINER, Stanley, B.** [US/US]; 400 Pacheco Street, San Francisco, CA

94116 (US). **SUPATTAPONE, Surachai** [US/US]; 225 Buckingham Way #702, San Francisco, CA 94132 (US). **SCOTT, Michael, R.** [GB/US]; 1200 Clayton Street #9, San Francisco, CA 94114 (US).

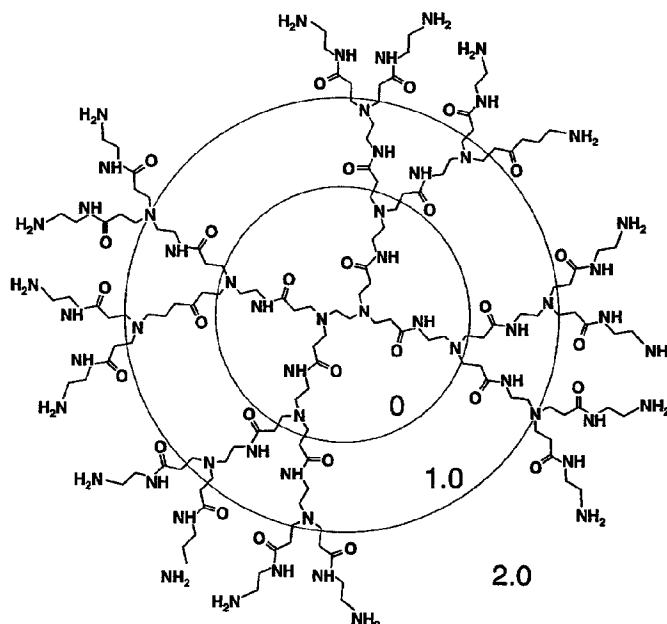
(74) Agent: **DEVORE, Dianna, L.**; Bozicevic, Field & Francis LLP, Suite 200, 200 Middlefield Road, Menlo Park, CA 94025 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: METHOD OF STERILIZING



(57) Abstract: A method of sterilizing objects as well as the sterilized objects obtained from the method are disclosed. The method involves contacting an object such as a medical device to be reused with polycationic dendrimer under conditions which result in rendering a conformationally altered protein (e.g. a prion) non-infectious. A disinfecting agent or surgical scrub composition which comprises the dendrimers is also disclosed as are gelatin capsules treated with polycationic dendrimers.

WO 00/72851 A1



Published:

— With international search report.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

METHOD OF STERILIZING

GOVERNMENT SUPPORT

This work was supported, in part, by grants from the National Institutes of Health NS14069,
5 AG08967, AG02132, AG10770 and K08 NS02048-02. The government may have certain rights in
this work.

FIELD OF THE INVENTION

The present invention relates generally to methods of sterilizing materials and particularly to
10 a method of inactivating infectious prions.

BACKGROUND OF THE INVENTION

There are large numbers of known methods of sterilizing materials. Many methods involve
heating a material to a temperature at which pathogens are killed or inactivated. Other methods
15 involve exposing the material to compounds which kill or inactivate pathogens which are contacted
by the compounds. Still other methods involve irradiating a material with a sufficient amount of a
particular type of radiation for a period of time sufficient to inactivate, disrupt or kill pathogens in
the material. These methods are generally directed toward killing bacteria and inactivating viruses
present in or on the material. Although sterilization methods may be quite effective in killing
20 bacteria or inactivating viruses, they do not generally inactivate pathogenic proteins such as prions
which can be responsible for a number of fatal diseases.

There are a considerable number of diseases associated with a conformationally altered
protein. For example, Alzheimer's disease is associated with APP, A β peptide, α 1-antichymotrypsin,
tau and non-A β component. Many of these diseases are neurological diseases. However, type II
25 Diabetes is associated with Amylin and Multiple myeloma-plasma cell dyscrasias is associated with
IgGL-chain. The relationship between the disease onset and the transition from the normal protein to
the conformationally altered protein has been examined very closely in some instances such as with
the association between prion diseases and PrP^{Sc}.

Prion diseases are a group of fatal neurodegenerative disorders that can occur in hereditary, sporadic, and infectious forms (Prusiner, S.B. Scrapie prions. *Annu. Rev. Microbiol.* **43**, 345-374 (1989)). These illnesses occur in humans and a variety of other animals (Prusiner, S.B. Prions. *Proc. Natl. Acad. Sci. USA* **95**, 13363-13383 (1998)). Prions are infectious proteins. The normal, cellular form of the prion protein (PrP) designated PrP^C contains three α -helices and has little β -sheet; in contrast, the protein of the prions denoted PrP^{Sc} is rich in β -sheet structure. The accumulation of PrP^{Sc} in the central nervous system (CNS) precedes neurologic dysfunction accompanied by neuronal vacuolation and astrocytic gliosis.

The spectrum of human prion diseases includes kuru (Gajdusek, D.C., Gibbs, C.J., Jr. & Alpers, M. Experimental transmission of a kuru-like syndrome to chimpanzees. *Nature* **209**, 794-796 (1966)), Creutzfeldt-Jakob disease (CJD) (Gibbs, C.J., Jr., *et al.* Creutzfeldt-Jakob disease (spongiform encephalopathy): transmission to the chimpanzee. *Science* **161**, 388-389 (1968)), Gerstmann-Sträussler-Scheinker disease (GSS) and fatal familial insomnia (FFI) (Goldfarb, L.G., *et al.* Fatal familial insomnia and familial Creutzfeldt-Jakob disease: disease phenotype determined by a DNA polymorphism. *Science* **258**, 806-808 (1992); Medori, R., *et al.* Fatal familial insomnia: a second kindred with mutation of prion protein gene at codon 178. *Neurology* **42**, 669-670 (1992)), and a new form of human prion disease, new variant CJD (nvCJD), which has emerged in Great Britain and France (Will, R.G., *et al.* A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet* **347**, 921-925 (1996); Cousens, S.N., Vynnycky, E., Zeidler, M., Will, R.G. & Smith, P.G. Predicting the CJD epidemic in humans. *Nature* **385**, 197-198 (1997); Will, R.G., *et al.* Deaths from variant Creutzfeldt-Jakob disease. *Lancet* **353**, 979 (1999)). Several lines of evidence have suggested a link between the nvCJD outbreak and a preceding epidemic of bovine spongiform encephalopathy (BSE) (Will, R.G., *et al.* A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet* **347**, 921-925 (1996); Bruce, M.E., *et al.* Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature* **389**, 498-501 (1997); Hill, A.F., *et al.* The same prion strain causes vCJD and BSE. *Nature* **389**, 448-450 (1997); Lasmézas, C.I., *et al.* BSE transmission to macaques. *Nature* **381**, 743-744 (1996)). Although it is too early to predict the number of nvCJD cases that might eventually arise in Great Britain and elsewhere (Cousens, S.N., Vynnycky, E., Zeidler, M., Will, R.G. & Smith, P.G. Predicting the CJD epidemic in humans. *Nature* **385**, 197-198 (1997)), it is clear that effective therapeutics for prion diseases are urgently needed. Unfortunately, although a number of compounds including amphotericins, sulfated polyanions, Congo red dye, and anthracycline antibiotics have been reported as prospective therapeutic agents (Ingrosso, L., Ladogana, A. & Pocchiari, M. Congo red prolongs the incubation period in scrapie-infected hamsters. *J. Virol.* **69**, 506-508 (1995); Tagliavini, F., *et al.*

Effectiveness of anthracycline against experimental prion disease in Syrian hamsters. *Science* **276**, 1119-1122 (1997); Masullo, C., Macchi, G., Xi, Y.G. & Pocchiari, M. Failure to ameliorate Creutzfeldt-Jakob disease with amphotericin B therapy. *J. Infect. Dis.* **165**, 784-785 (1992); Ladogana, A., *et al.* Sulphate polyanions prolong the incubation period of scrapie-infected hamsters. *J. Gen. Virol.* **73**, 661-665 (1992)), all have demonstrated only modest potential to impede prion propagation, and none have been shown to effect the removal of pre-existing prions from an infected host.

The PrP gene of mammals expresses a protein which can be the soluble, non-disease form PrP^C or be converted to the insoluble, disease form PrP^{Sc}. PrP^C is encoded by a single-copy host gene [Basler, Oesch *et al.* (1986) *Cell* 46:417-428] and when PrP^C is expressed it is generally found on the outer surface of neurons. Many lines of evidence indicate that prion diseases result from the transformation of the normal form of prion protein (PrP^C) into the abnormal form (PrP^{Sc}). There is no detectable difference in the amino acid sequence of the two forms. However, PrP^{Sc} when compared with PrP^C has a conformation with higher β -sheet and lower α -helix content (Pan, Baldwin *et al.* (1993) *Proc Natl Acad Sci USA* 90:10962-10966; Safar, Roller *et al.* (1993) *J Biol Chem* 268:20276-20284). The presence of the abnormal PrP^{Sc} form in the brains of infected humans or animals is the only disease-specific diagnostic marker of prion diseases.

PrP^{Sc} plays a key role in both transmission and pathogenesis of prion diseases (spongiform encephalopathies) and it is a critical factor in neuronal degeneration (Prusiner (1997) *The Molecular and Genetic Basis of Neurological Disease*, 2nd Edition : 103-143). The most common prion diseases in animals are scrapie of sheep and goats and bovine spongiform encephalopathy (BSE) of cattle (Wilesmith and Wells (1991) *Curr Top Microbiol Immunol* 172:21-38). Four prion diseases of humans have been identified: (1) kuru, (2) Creutzfeldt-Jakob Disease (CJD), (3) Gerstmann-Sträussler-Scheinker Disease (GSS), and (4) fatal familial insomnia (FFI) [Gajdusek (1977) *Science* 197:943-960; Medori, Tritschler *et al.* (1992) *N Engl J Med* 326:444-449]. Initially, the presentation of the inherited human prion diseases posed a conundrum which has since been explained by the cellular genetic origin of PrP.

The assembly and misassembly of normally soluble proteins into conformationally altered proteins is thought to be a causative process in a variety of other diseases. Structural conformational changes are required for the conversion of a normally soluble and functional protein into a defined, insoluble state. Examples of such insoluble protein include: A β peptide in amyloid plaques of Alzheimer's disease and cerebral amyloid angiopathy (CAA); α -synuclein deposits in Lewy bodies of Parkinson's disease, tau in neurofibrillary tangles in frontal temporal dementia and Pick's disease; superoxide dismutase in amyotrophic lateral sclerosis; huntingtin in Huntington's disease; and prions

in Creutzfeldt-Jakob disease (CJD): (for reviews, see Glenner et al. (1989) *J. Neurol. Sci.* 94:1-28; Haan et al. (1990) *Clin. Neurol. Neurosurg.* 92(4):305-310).

Often these highly insoluble proteins form aggregates composed of nonbranching fibrils with the common characteristic of a β -pleated sheet conformation. In the CNS, amyloid can be present in cerebral and meningeal blood vessels (cerebrovascular deposits) and in brain parenchyma (plaques). Neuropathological studies in human and animal models indicate that cells proximal to amyloid deposits are disturbed in their normal functions (Mandybur (1989) *Acta Neuropathol.* 78:329-331; Kawai et al. (1993) *Brain Res.* 623:142-6; Martin et al. (1994) *Am. J. Pathol.* 145:1348-1381; Kalaria et al. (1995) *Neuroreport* 6:477-80; Masliah et al. (1996) *J. Neurosci.* 16:5795-5811).

Other studies additionally indicate that amyloid fibrils may actually initiate neurodegeneration (Lendon et al. (1997) *J. Am. Med. Assoc.* 277:825-31; Yankner (1996) *Nat. Med.* 2:850-2; Selkoe (1996) *J. Biol. Chem.* 271:18295-8; Hardy (1997) *Trends Neurosci.* 20:154-9).

In both AD and CAA, the main amyloid component is the amyloid β protein ($A\beta$). The $A\beta$ peptide, which is generated from the amyloid β precursor protein (APP) by two putative secretases, is present at low levels in the normal CNS and blood. Two major variants, $A\beta_{1-40}$ and $A\beta_{1-42}$, are produced by alternative carboxy-terminal truncation of APP (Selkoe et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:7341-7345; Selkoe, (1993) *Trends Neurosci* 16:403-409). $A\beta_{1-42}$ is the more fibrillogenic and more abundant of the two peptides in amyloid deposits of both AD and CAA. In addition to the amyloid deposits in AD cases described above, most AD cases are also associated with amyloid deposition in the vascular walls (Hardy (1997), *supra*; Haan et al. (1990), *supra*; Terry et al., *supra*; Vinters (1987), *supra*; Itoh et al. (1993), *supra*; Yamada et al. (1993), *supra*; Greenberg et al. (1993), *supra*; Levy et al. (1990), *supra*). These vascular lesions are the hallmark of CAA, which can exist in the absence of AD.

Human transthyretin (TTR) is a normal plasma protein composed of four identical, predominantly β -sheet structured units, and serves as a transporter of hormone thyroxine. Abnormal self assembly of TTR into amyloid fibrils causes two forms of human diseases, namely senile systemic amyloidosis (SSA) and familial amyloid polyneuropathy (FAP) (Kelly (1996) *Curr Opin Struc Biol* 6(1):11-7). The cause of amyloid formation in FAP are point mutations in the TTR gene; the cause of SSA is unknown. The clinical diagnosis is established histologically by detecting deposits of amyloid in situ in bioptic material.

To date, little is known about the mechanism of TTR conversion into amyloid *in vivo*. However, several laboratories have demonstrated that amyloid conversion may be simulated *in vitro* by partial denaturation of normal human TTR [McCutchen, Colon et al. (1993) *Biochemistry* 32(45):12119-27; McCutchen and Kelly (1993) *Biochem Biophys Res Commun* 197(2) 415-21]. The mechanism of conformational transition involves monomeric conformational intermediate which

polymerizes into linear β -sheet structured amyloid fibrils [Lai, Colon et al. (1996) *Biochemistry* 35(20):6470-82]. The process can be mitigated by binding with stabilizing molecules such as thyroxine or triiodophenol (Mirov, Lai et al. (1996) *Proc Natl Acad Sci USA* 93(26):15051-6).

5 The precise mechanisms by which neuritic plaques are formed and the relationship of plaque formation to the disease-associated neurodegenerative processes are not well-defined. The amyloid fibrils in the brains of Alzheimer's and prion disease patients are known to result in the inflammatory activation of certain cells. For example, primary microglial cultures and the THP-1 monocytic cell line are stimulated by fibrillar β -amyloid and prion peptides to activate identical tyrosine kinase-dependent inflammatory signal transduction cascades. The signaling response elicited by
10 β -amyloid and prion fibrils leads to the production of neurotoxic products, which are in part responsible for the neurodegenerative . C.K. Combs et al, *J Neurosci* 19:928-39 (1999).

Although research efforts relating to conformationally altered proteins are advancing efforts to sterilize materials to avoid infections with such proteins are not keeping pace. The present invention offers a means of sterilizing materials which contain conformationally altered proteins such
15 as prions.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a schematic drawing of a dendrimer molecule showing the defined "generations" of homodisperse structure created using a repetitive divergent growth technique. The specific
20 diagram is of PAMAM, generation 2.0 (ethylene diamine core).

SUMMARY OF THE INVENTION

A method is disclosed whereby any type of object can be sterilized by combining normal sterilization procedures with the use of a polycationic dendrimer which is capable of rendering a
25 conformationally altered protein such as a prion non-infectious. The method is particularly useful in sterilizing medical devices such as surgical instruments and catheters which have been used and brought into contact with blood or brain tissue. Objects sterilized via the method are also part of the invention and include capsules which are made from gelatin extracted from cattle which cattle may be infected with prions, i.e. have undiagnosed BSE known as "mad cow disease." The polycationic
30 dendrimers can be combined with conventional antibacterial and antiviral agents in aqueous or alcohol solutions to produce disinfecting agents or surgical scrubs. Branched polycations for use in the invention include, but are not limited to, polypropylene imine, polyethyleneimine (PEI) poly(4'-aza-4'-methylheptamethylene D-glucaramide), polyamidoamines and suitable fragments and/or variants of these compounds.

An aspect of the invention is a method of treating objects with a composition characterized by its ability to render proteins associated with diseases non-infectious.

5 An advantage of the invention is that proteins such as prions can be rendered non-infectious without the need for extreme conditions such as exposure to heat over long periods of time, e.g. 1-10 hours at 100°-200°C.

A feature of the invention is that compositions can be useful while containing only very low concentrations of polycationic dendrimers, e.g. 1% to 0.001%.

Another aspect of the invention is that capsules made with bovine gelatin can be certified prion free.

10 Another aspect of the invention is that drugs produced from cell cultures treated with polycationic dendrimers can be certified prion free.

Still another aspect of the invention is that medical devices being reused after exposure to blood or brain tissue can be certified prion free.

15 Still another aspect of the invention is that hospitals, operating rooms and the devices and equipment within them can be certified prion free by contacting them with polycationic dendrimers at standard temperatures and pressures. A pharmaceutical composition for the treatment of insoluble protein deposit formation in an animal, said composition comprising a therapeutically effective amount of a branched polycation; and a pharmaceutically acceptable excipient. In one embodiment, the branched polycation is a branched polymer, and wherein at least one branch is
20 positively charged, and the branched polymer may have multiple charged branches. The branches may have the same chemical structure, or the branches may vary in structure within a single molecule. Examples of polymers that may be used in such a pharmaceutical composition include polypropylene imine, polyethyleneimine (PEI) poly(4'-aza-4'-methylheptamethylene D-glucaramide), polyamidoamines and pharmaceutically effective variants or fragments thereof. In
25 one embodiment of the pharmaceutical composition, the composition also contains a second therapeutic agent, such as an analgesic agent, an antimicrobial agent, anti-inflammatory agent, an antioncogenic agent, an antiviral agent, and the like.

The present invention also provides a method of enhancing clearance of a disease related conformation of a protein from cells by contacting cells with a branched polycation for a time
30 sufficient to enhance the rate of clearance of a disease related conformation of a protein from the cells. This branched polycation can be administered *in vivo* or *ex vivo* to a subject including a human, cow, sheep, deer, dog, cat, goat, chicken and turkey. Examples of such disease related proteins include PrP^{Sc}, APP, A β peptide, α -1-antichymotrypsin. The method is thus useful for subjects suffering from disorders such as bovine spongiform encephalopathy,

Creutzfeldt-Jacob Disease, fatal familial insomnia, GSS for Gerstmann-Straussler-Scheinker Disease, kuru, scrapie, Alzheimer's Disease, Frontal temporal dementia, Huntington's disease, ALS, Pick's disease, Parkinson's disease, Diabetes Type II, multiple myeloma, familial amyloidotic polyneuropathy, medullary carcinoma of thyroid, chronic renal failure, congestive heart failure, senile cardiac and systemic amyloidosis, chronic inflammation, atherosclerosis, and familial amyloidosis. The branched polycation can be administered to a subject in an amount non-toxic to the subject, for example a dosage of 0.001 mg to 1 mg/kg body weight per day. The polycation may be administered in a single dosage form, or it may be repeatedly administered to the subject. The branched polycation may also be administered prophylactically to prevent the formation of the disease conformation of these proteins.

The invention also provides a food composition for the preventing insoluble protein deposit formation in an animal, where the food contains a therapeutically effective amount of a branched cation which enhances clearance of conformationally altered protein. The food can be any food product, including solid foods such as meat (*e.g.*, beef or lamb) and liquid foods such as vinegar, oil, and condiments such as steak sauce and ketchup. The branched polycation is allowed to contact the food prior to ingestion for a time sufficient to allow clearance of the conformationally altered proteins.

The present invention also provides a method of preventing a farm animal from acquiring a disease associated with a conformationally altered protein by feeding the animals animal feed containing a branched polycation. The feed containing the branched polycation may be synthetically produced, and fed directly to the animals, or the branched polycation may be introduced to a natural food source, *e.g.*, the animal feed is grass and the branched polycation is sprayed on the grass or introduced to the grass through a plant fertilizer. One aspect of this embodiment of the invention is a method of preventing disease caused by ingestion of contaminated food products by feeding animals foods containing branched polycations.

The present invention also provides a method of enhancing clearance of a disease related conformation of a protein from a meat food product by contacting the meat with a compound which enhances clearance of a conformationally altered protein at a pH of 5 or less for a time sufficient to allow for destruction of conformationally altered protein.

An advantage of the invention is that conformationally altered protein such as prions can be rendered non-infectious with a method which need only consist of applying a polycationic dendrimer preferably held at a pH of 5.0 or less.

Another aspect of the invention is soaps, surgical scrubs, detergents and the like with polycationic dendrimers therein.

An advantage of the invention is that compositions containing polycationic dendrimers can be used to inactivate prions which might be present on surgical instruments, knives and/or other tools or equipment used by butchers, particularly those used in the butchering of cows or other animals which might be infected with prions.

A feature of the invention is that compositions of the invention can be effective in activating prions when the polycationic dendrimers are present in very low concentrations, e.g. 1% to 0.001% or less.

These and other aspects, advantages, and features of the invention will become apparent to those persons skilled in the art upon reading the details of the compounds, and assay method more fully described below.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Before the present methods, objects and compositions are described, it is to be understood that this invention is not limited to the particular steps, devices or components described and, as such, may of course vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

DEFINITIONS

The term "detergent" is used to mean any substance that reduces the surface tension of water. The detergent may be a surface active agent which concentrates at oil-water interfaces, exerts emulsifying action and thereby aids in removing soils e.g. common sodium soaps of fatty acids. A detergent may be anionic, cationic, or monionic depending on their mode of chemical action.

Detergents include linear alkyl sulfonates (LAS) often aided by "builders." A LAS is preferably an

alkyl benzene sulfonate ABS which is readily decomposed by microorganisms (biodegradable). The LAS is generally a straight chain alkyl comprising 10 to 30 carbon atoms. The detergent may be in a liquid or a solid form.

The term "conformationally altered protein" is used here to describe any protein which has a three dimensional conformation associated with a disease. The conformationally altered protein may cause the disease, be a factor in a symptom of the disease or appear as a result of other factors. The conformationally altered protein appears in another conformation which has the same amino acid sequence. In general, the conformationally altered protein formed is "constricted" in conformation as compared to the other "relaxed" conformation which is not associated with disease. The following is a non-limiting list of diseases with associated proteins which assemble two or more different conformations wherein at least one conformation is an example of a conformationally altered protein.

<u>Disease</u>	<u>Insoluble Proteins</u>
Alzheimer's Disease	APP, A β peptide, α 1-antichymotrypsin, tau, non-A β component, presenillin 1, presenillin 2 apoE
Prion diseases, Creutzfeldt Jakob disease, scrapie and bovine spongiform encephalopathy	PrP ^{Sc}
ALS	SOD and neurofilament
Pick's disease	Pick body
Parkinson's disease	α -synuclein in Lewy bodies
Frontotemporal dementia	tau in fibrils
Diabetes Type II	Amylin
Multiple myeloma-- plasma cell dyscrasias	IgGL-chain
Familial amyloidotic polyneuropathy	Transthyretin
Medullary carcinoma of thyroid	Procalcitonin
Chronic renal failure	β_2 --microglobulin
Congestive heart failure	Atrial natriuretic factor
Senile cardiac and systemic amyloidosis	Transthyretin
Chronic inflammation	Serum amyloid A

Atherosclerosis	ApoA1
Familial amyloidosis	Gelsolin
Huntington's disease	Huntingtin

5 The term "acid" is used to describe any compound or group of compounds which has one or more characteristics of (a) sour taste; (b) turns litmus dye red; (c) reacts with certain metals to form a salt; (d) reacts with certain bases or alkalines to form a salt. An acid comprises hydrogen and in water undergoes ionization so that H_3O^+ ions are formed - also written as H^+ and referred to as hydronium ions or simply hydrogen ions. Weak acids such as acetic acid or carbonic acid may be used as may strong acids such as hydrochloric acid, nitric acid and sulfuric acid. In compositions of the invention the acid is preferably present in a concentration so as to obtain a pH of 5 or less, more preferably 4 or less and still more preferably 3.5 ± 1 .

15 The terms "sterilizing", "making sterile" and the like are used here to mean rendering something non-infectious or rendering something incapable of causing a disease. Specifically, it refers to rendering a protein non-infectious or incapable of causing a disease or the symptoms of a disease. Still more specifically, it refers to rendering a conformationally altered protein (e.g. PrP^{Sc} known as prions) incapable of causing a disease or the symptoms of a disease.

20 By "effective dose" or "amount effective" is meant an amount of a compound sufficient to provide the desired sterilizing result. This will vary depending on factors such as the type of object or material being sterilized and the amount or concentration of infectious proteins which might be present. Polycations of the invention or more specifically polycationic dendrimer compounds of the invention could be mixed with a material in an amount in a range 1 to 500 μg of dendrimer per ml or mg of material being sterilized. The concentration is sufficient if the resulting composition is effective in decreasing the infectivity of conformationally altered proteins such that the treated material over time would not result in infection. Because (1) some materials will have higher concentrations of altered protein than others (2) some materials are contacted more frequently than others and (3) individual proteins have different degrees of infectivity the effective dose or concentration range needed to sterilize can vary considerably. It is also pointed out that the dose needed to treat an amount of material may vary somewhat based on the pH the treatment is carried out at and the amount of time the compound is maintained in contact with the material at the desired low pH (e.g., 5.0 or less) level.

35 The term "LD₅₀" as used herein is the dose of an active substance that will result in 50 percent lethality in all treated experimental animals. Although this usually refers to invasive administration, such as oral, parenteral, and the like, it may also apply to toxicity using less invasive methods of administration, such as topical applications of the active substance.

The term "amine-terminated" includes primary, secondary and tertiary amines.

The terms "PrP protein", "PrP" and like are used interchangeably herein and shall mean both the infectious particle form PrP^{Sc} known to cause diseases (spongiform encephalopathies) in humans and animals and the noninfectious form PrP^C which, under appropriate conditions is converted to the infectious PrP^{Sc} form.

The terms "prion", "prion protein", "PrP^{Sc} protein" and the like are used interchangeably herein to refer to the infectious PrP^{Sc} form of a PrP protein, and is a contraction of the words "protein" and "infection." Particles are comprised largely, if not exclusively, of PrP^{Sc} molecules encoded by a PrP gene. Prions are distinct from bacteria, viruses and viroids. Known prions infect animals to cause scrapie, a transmissible, degenerative disease of the nervous system of sheep and goats, as well as bovine spongiform encephalopathy (BSE), or "mad cow disease", and feline spongiform encephalopathy of cats. Four prion diseases known to affect humans are (1) kuru, (2) Creutzfeldt-Jakob Disease (CJD), (3) Gerstmann-Sträussler-Scheinker Disease (GSS), and (4) fatal familial insomnia (FFI). As used herein "prion" includes all forms of prions causing all or any of these diseases or others in any animals used - and in particular in humans and domesticated farm animals.

The term "PrP gene" is used herein to describe genetic material which expresses proteins including known polymorphisms and pathogenic mutations. The term "PrP gene" refers generally to any gene of any species which encodes any form of a prion protein. Some commonly known PrP sequences are described in Gabriel et al., *Proc. Natl. Acad. Sci. USA* 89:9097-9101 (1992) and U.S. Patent No. 5,565,186, incorporated herein by reference to disclose and describe such sequences. The PrP gene can be from any animal, including the "host" and "test" animals described herein and any and all polymorphisms and mutations thereof, it being recognized that the terms include other such PrP genes that are yet to be discovered. The protein expressed by such a gene can assume either a PrP^C (non-disease) or PrP^{Sc} (disease) form.

The terms "standardized prion preparation", "prion preparation", "preparation" and the like are used interchangeably herein to describe a composition (e.g., brain homogenate) obtained from the brain tissue of mammals which exhibits signs of prion disease: the mammal may (1) include a transgene as described herein; (2) have an ablated endogenous prion protein gene; (3) have a high number of prion protein gene from a genetically diverse species; and/or (4) be a hybrid with an ablated endogenous prion protein gene and a prion protein gene from a genetically diverse species. Different combinations of 1-4 are possible, e.g., 1 and 2. The mammals from which standardized prion preparations are obtained exhibit clinical signs of CNS dysfunction as a result of inoculation with prions and/or due to developing the disease of their genetically modified make up, e.g., high copy number of prion protein genes. Standardized prion preparations and methods of making such

are described and disclosed in U.S. Patent 5,908,969 issued June 1, 1999 and application serial no. 09/199,523 filed November 25, 1998 both of which are incorporated herein by reference in their entirety to disclose and describe standardized prion preparations.

The term "Alzheimer's disease" (abbreviated herein as "AD") as used herein refers to a condition associated with formation of neuritic plaques comprising amyloid β protein, primarily in the hippocampus and cerebral cortex, as well as impairment in both learning and memory. "AD" as used herein is meant to encompass both AD as well as AD-type pathologies.

The term "AD-type pathology" as used herein refers to a combination of CNS alterations including, but not limited to, formation of neuritic plaques containing amyloid β protein in the hippocampus and cerebral cortex. Such AD-type pathologies can include, but are not necessarily limited to, disorders associated with aberrant expression and/or deposition of APP, overexpression of APP, expression of aberrant APP gene products, and other phenomena associated with AD. Exemplary AD-type pathologies include, but are not necessarily limited to, AD-type pathologies associated with Down's syndrome that is associated with overexpression of APP.

The term "phenomenon associated with Alzheimer's disease" as used herein refers to a structural, molecular, or functional event associated with AD, particularly such an event that is readily assessable in an animal model. Such events include, but are not limited to, amyloid deposition, neuropathological developments, learning and memory deficits, and other AD-associated characteristics.

The term "cerebral amyloid angiopathy" (abbreviated herein as CAA) as used herein refers to a condition associated with formation of amyloid deposition within cerebral vessels which can be complicated by cerebral parenchymal hemorrhage. CAA is also associated with increased risk of stroke as well as development of cerebellar and subarachnoid hemorrhages (Vinters (1987) *Stroke* 18:311-324; Haan et al. (1994) *Dementia* 5:210-213; Itoh et al. (1993) *J. Neurol. Sci.* 116:135-414). CAA can also be associated with dementia prior to onset of hemorrhages. The vascular amyloid deposits associated with CAA can exist in the absence of AD, but are more frequently associated with AD.

The term "phenomenon associated with cerebral amyloid angiopathy" as used herein refers to a molecular, structural, or functional event associated with CAA, particularly such an event that is readily assessable in an animal model. Such events include, but are not limited to, amyloid deposition, cerebral parenchymal hemorrhage, and other CAA-associated characteristics.

The term " β -amyloid deposit" as used herein refers to a deposit in the brain composed of A β as well as other substances.

Abbreviations used herein include:

- CNS for central nervous system;
BSE for bovine spongiform encephalopathy;
CJD for Creutzfeldt-Jakob Disease;
5 FFI for fatal familial insomnia;
GSS for Gerstmann-Sträussler-Scheinker Disease;
AD for Alzheimer's disease;
CAA for cerebral amyloid angiopathy;
Hu for human;
10 HuPrP for human prion protein;
Mo for mouse;
MoPrP for mouse prion protein;
SHa for a Syrian hamster;
SHaPrP for a Syrian hamster prion protein;
15 PAMAM for polyamidoamide dendrimers
PEI for polyethyleneimine
PPI for polypropyleneimine
PrP^{Sc} for the scrapie isoform of the prion protein;
PrP^C for the cellular contained common, normal isoform of the prion protein;
20 PrP 27-30 or PrP^{Sc} 27-30 for the treatment or protease resistant form of PrP^{Sc};
MoPrP^{Sc} for the scrapie isoform of the mouse prion protein;
N2a for an established neuroblastoma cell line used in the present studies;
ScN2a for a chronically scrapie-infected neuroblastoma cell line;
ALS for amyotrophic lateral sclerosis;
25 HD for Huntington's disease;
FTD for frontotemporal dementia;
SOD for superoxide dismutase

GENERAL ASPECTS OF THE INVENTION

- 30 The invention comprises compositions of compounds found to be effective in rendering conformationally altered proteins non-infective. The compositions are preferably low pH solutions comprised of a non-toxic weak acid such as acetic acid having dissolved therein a branched polycation. Preferred compositions of the invention are in the form of aqueous or alcohol solutions which are comprised of a branched polycation, an antibacterial, an antifungal and an antiviral
35 compound. The compositions are coated on, mixed with, injected into or otherwise brought into

contact with a material to be sterilized. The composition is applied in a manner so that the branched polycation is maintained at a low pH (e.g. 5 or less and preferably 3.5 ± 1) in an amount of 1 μg or more polycation per ml or mg of material to be sterilized. The composition is maintained in the desired pH range at normal temperature (e.g., 15°C to 30°C) for a sufficient period of time (e.g. 1
5 hour to 1 week) to cause conformationally altered protein present on or in the material to be destroyed (e.g. hydrolyzed) or rendered non-infective. Preferred compositions of the invention are useful in cleaning and sterilizing and may be comprised of a polycationic dendrimers, a detergent, and an acid providing a pH of about 3.5 ± 1 .

10 DENDRIMER COMPOUNDS WHICH CLEAR PRIONS

Dendrimers are branched compounds also known as "starburst" or "star" polymers due to a characteristic star-like structure (see Figure 1). Dendrimers of the invention are polymers with structures built from AB_n monomers, with $n \geq 2$, and preferably $n=2$ or 3. Such dendrimers are highly branched and have three distinct structural features: 1) a core, 2) multiple peripheral
15 end-groups, and 3) branching units that link the two. Dendrimers may be cationic (full generation dendrimers) or anionic (half generation dendrimers). For a review on the general synthesis, physical properties, and applications of dendrimers, see, e.g., Tomalia et. al, *Angew. Chem. Int. Ed. Engl.*, 29:138-175, (1990); Y. Kim and C. Zimmerman, *Curr Opin Chem Biol*, 2:733-7421 (1997).

20 In a preferred embodiment, sterilizing compositions of the invention comprise a cationic dendrimer preferably dissolved in a low pH solvent such as acetic acid. Examples of suitable dendrimers are disclosed in U.S. Pat. Nos. 4,507,466, 4,558,120, 4,568,737, 4,587,329, 4,631,337, 4,694,064, 4,713,975, 4,737,550, 4,871,779, and 4,857,599 to D. A. Tomalia, et al., which are hereby incorporated by reference to disclose and describe such compounds.

25 Dendrimers typically have tertiary amines which have a pKa of 5.7. The dendrimers can optionally be chemically or heat treated to remove some of the tertiary amines. Other suitable cations include polypropylene imine, polyethyleneimine (PEI), which has tertiary amines with a pKa of 5.9, and poly(4'-aza-4'-methylheptamethylene D-glucaramide), which has tertiary amines with a pKa of 6.0. The cationic dendrimer is preferably dissolved in the low pH solvent such as
30 vinegar in a concentration of 0.0001% or more, preferably 0.01% or more and more preferably about 1%.

Preferably, the dendrimers for use in the invention are polyamidoamines (hereinafter "PAMAM"). PAMAM dendrimers are particularly biocompatible, since polyamidoamine groups resemble peptide bonds of proteins.

Dendrimers are prepared in tiers called generations (see generations 0, 1 and 2 in Figure 1) and therefore have specific molecular weights. The full generation PAMAM dendrimers have amine terminal groups, and are cationic, whereas the half generation dendrimers are carboxyl terminated. Full generation PAMAM dendrimers are thus preferred for use in the present invention. PAMAM dendrimers may be prepared having different molecular weights and have specific values as described in Table 1 below for generations 0 through 10.

TABLE A
LIST OF PAMAM DENDRIMERS AND THEIR
MOLECULAR WEIGHTS (Ethylene Diamine core, amine terminated),

	<u>GENERATION</u>	<u>TERMINAL GROUPS</u>	<u>MOL. WT. g/mole</u>
	0	4	517
	1	8	1430
15	2	216	3256
	3	32	6909
	4	64	14,215
	5	128	28,795
	6	256	58,048
20	7	512	116,493
	8	1024	233,383
	9	2048	467,162
	10	4096	934,720

As shown in Table A, the number of terminal amine groups for PAMAM dendrimers generations 0 through 10 range from 4 to 4,096, with molecular weights of from 517 to 934,720. PAMAM dendrimers are available commercially from Aldrich or Dendritech. Polyethyleneimine or polypropylene dendrimers or quaternized forms of amine-terminated dendrimers may be prepared as described by Tomalia et. al, *Angew. Chem. Int. Ed. Engl.*, 29:138-175 (1990) incorporated by reference to describe and disclose methods of making dendrimers.

STERILIZING COMPOSITIONS

Examples provided here show that highly-branched polycations, e.g. dendrimer compounds, affect the extent and distribution of PrP^{Sc} protein deposits in scrapie-infected cells. The presence of dendrimers in a low pH environment and at relatively low, non-cytotoxic levels results in a significant reduction in detectable PrP^{Sc} in cells and brain homogenates. Thus, the present invention encompasses compositions for reducing, inhibiting, or otherwise mitigating the degree of infectivity of a protein. A composition of the invention is comprised of any compound

capable of destroying conformationally altered proteins when in a low pH environment, (e.g. a polycationic dendrimer) in solution, suspension or mixture.

STERILIZING FORMULATIONS

Sterilizing compositions of the invention preferably contain highly branched polycations, e.g. polycationic dendrimer, in a concentration from 0.0001 to 10% of the formulation. The following methods and excipients are merely exemplary and are in no way limiting.

In addition to including the compound such as a highly branched cationic compound in the formulation it is important to maintain that compound in a low pH environment. Any number of known acids or mixtures of acids could be used with the invention. Non-limiting examples of commercially available products which could be supplemented with the cationic compounds are described below. In these formulations the percentage amount of each ingredient can vary. In general a solvent ingredient (e.g. water or alcohol) is present in amounts of 40% to 100% and the last listed ingredient is present in a range of 0.5% to 5%. The other ingredients are present in an amount in a range of 1% to 60% and more generally 5% to 20%. In each case the polycationic compounds of the invention are added in amounts of about 0.01% to 5% and preferably 0.1% to 2% and more preferably about 1%. The amount added is an amount needed to obtain the desired effect.

FORMULATION 1

Component	wt %
acid	90 - 99.99
polycationic dendrimer	0.01 - 10

FORMULATION 2

Component	wt %
water	10 - 99
acid	1 - 20
polycationic dendrimer	0.01 - 10

FORMULATION 3

Component	wt %
water	10 - 98
acid	1 - 20
detergent	1 - 20
polycationic dendrimer	0.01 - 5

5

FORMULATION 4	
Component	wt %
water	10 - 98
acetic acid	1 - 20
linear alkyl sulfonate	1 - 20
polycationic dendrimer	0.01 - 5

10

FORMULATION 5	
Component	wt %
water	1 - 98
alcohol	0 - 98
acid	1 - 20
detergent	1 - 20
polycationic dendrimer	0.1 - 5

15

20

FORMULATION 6	
Component	wt %
water	1 - 99
acid	1 - 20
antibacterial	0.1 - 5
detergent	1 - 20
polycationic dendrimer	0.1 - 5

25

30

FORMULATION 7	
Component	wt %
water	3 - 98.889
antimicrobial active agent	0.001- 5
anionic surfactant	1 - 80
protein donating agent	0.1 - 12
polycationic dendrimer	0.01 - 5

35

40

FORMULATION 8	
Component	wt %
Polycationic Dendrimer	0.5
Ethanol	74.0
Benzalkonium chloride	0.2
CAE	0.02
Glycerine	1.0
Chain silicone	0.5
Triglyceride	0.5
Lactic acid	10.0
Purified water	13.28

FORMULATION 9

Component	wt %
Polycationic Dendrimer	1.0
Ethanol	75.0
Benzalkonium chloride	0.2
CAE	0.02
Glycerine	1.0
Cyclic silicone	0.2
Triglyceride	0.3
Acetic Acid	20.0
Purified water	2.28

FORMULATION 10

Component	wt %
Polycationic Dendrimer	0.25
Ethanol	74.0
Chlorhexedine gluconate	0.75
Benzalkonium chloride	0.2
CAE	0.02
Glycerine	2.0
Chain silicone	0.2
Cyclic silicone	0.2
Triglyceride	0.3
Acetic Acid	20.0
Purified water	2.08

FORMULATION 11

Component	wt %
Polycationic Dendrimer	0.1
Ethanol	75.0
Chlorhexedine gluconate	0.9
Benzalkonium chloride	0.2
CAE	0.02
Glycerine	1.0
Chain silicone	0.5
Cyclic silicone	0.5
Triglyceride	0.3
Lactic acid	14.0
Purified water	7.98

FORMULATION 12

Component	wt %
Polycationic Dendrimer	0.01
Ethanol	75.0
Benzalkonium chloride	0.2
CAE	0.02
Glycerine	2.0
Chain silicone	0.99
Cyclic silicone	2.0
Triglyceride	3.0
Lactic acid	9
Purified water	7.78

FORMULATION 13

Component	wt %
Polycationic Dendrimer	1
Ethanol	75.0
Chlorhexedine gluconate	0.2
Benzalkonium chloride	0.2
CAE	0.02
Glycerine	0.8
Chain silicone	0.2
Cyclic silicone	0.2
Triglyceride	.38
Acetic acid	10
Purified water	12

FORMULATION 14

Component	wt %
Polycationic Dendrimer	0.001
Ethanol	75.99
Chlorhexedine gluconate	0.2
CAE	0.02
Glycerine	1.0
Chain silicone	0.2
Triglyceride	0.3
Lactic acid	14
Purified water	8.28

FORMULATION 15	
Component	wt %
Polycationic Dendrimer	1
Ethanol	75.0
Benzalkonium chloride	0.2
CAE	0.02
1, 3-butylene glycol	1.0
Methylphenyl polysiloxane	0.2
Isopropyl myristate (IPM)	0.3
Purified water	22.28

By using the disclosure provided here and other information such as taught in U.S. Patents 5,767,054; 6,007,831; 5,830,488; 5,968,539; 5,416,075; 5,296,158; and patents and publications cited therein those skilled in the art can produce countless other formulations of the invention. Further, such formulations can be used as described in such publications and can be packaged in any suitable container or dispenser device, e.g. taught in 5,992,698.

Formulations of the invention used with a cell culture have the advantage that they are non-toxic. For example, parenteral administration of a solution of the formulations of the invention is preferably nontoxic at a dosage of 0.1 mg/mouse, which is an LD₅₀ of less than one at 40 mg/Kg. Various nutrient formulations and/or injectable formulations of the type known to those skilled in the art can be used to prepare formulations for treating cell cultures.

Those skilled in the art will understand that in some situations it may be desirable to further reduce the pH environment to obtain the desired results. This can be accomplished by adding any desired acid. If desired, the pH can be raised to a normal level after treatment is complete, i.e. after a sufficient amount of any conformationally altered protein present are destroyed.

Compounds effective in sterilizing compositions containing conformationally altered proteins are determined via a cell culture assay and an organ homogenate assay each of which is described below in detail.

ScN2a CELL BASED ASSAY

Efforts were made to optimize the transfection of ScN2a cells with pSPOX expression plasmids (Scott, M.R., Köhler, R., Foster, D. & Prusiner, S.B. Chimeric prion protein expression in cultured cells and transgenic mice. *Protein Sci.* 1, 986-997 (1992)). In connection with those effects an evaluation was made of a transfection protocol that used SuperFect reagent (QIAGEN®). It was found that epitope-tagged (MHM2) PrP^{Sc} (Scott, M.R., Köhler, R., Foster, D. & Prusiner, S.B. Chimeric prion protein expression in cultured cells and transgenic mice. *Protein Sci.* 1, 986-997

(1992)) could not be detected in ScN2a cells following SuperFect-mediated transfection, whereas MHM2 PrP^{Sc} was efficiently formed when a cationic liposome method for DNA delivery was used. Close scrutiny revealed that, prior to protease digestion, SuperFect-transfected samples expressed MHM2 bands, which are not seen in the background pattern of an untransfected sample. The 3F4
5 monoclonal antibody does not react with MoPrP but does exhibit high background staining on Western blots of mouse ScN2a cells. Increased immunostaining in the 20-30 kDa region was observed compared to the non-transfected sample. These observations led us to conclude that MHM2 PrP was successfully expressed using SuperFect transfection reagent, but that conversion of MHM2 PrP^C to protease-resistant MHM2 PrP^{Sc} was inhibited by SuperFect.

10 To investigate this apparent inhibition, a Western blot was reprobed with RO73 polyclonal antiserum to detect endogenous MoPrP^{Sc}, the presence of which is diagnostic for prion infection in ScN2a cells (Butler, D.A., *et al.* Scrapie-infected murine neuroblastoma cells produce protease-resistant prion proteins. *J. Virol.* **62**, 1558-1564 (1988)). Surprisingly, it was found that the SuperFect-treated ScN2a cells no longer contained detectable quantities of MoPrP^{Sc} - also confirmed
15 in Western blots. To investigate the mechanism by which SuperFect reduced the level of pre-existing PrP^{Sc} in chronically infected ScN2a cells, measurements were made of endogenous PrP^{Sc} in ScN2a cells exposed to various concentrations of SuperFect in the absence of plasmid DNA. The results showed that treatment with SuperFect (a branched polycation) caused the disappearance of PrP^{Sc} from ScN2a cells in a dose-dependent manner. The concentration of SuperFect required to eliminate
20 >95% of pre-existing PrP^{Sc} with a three hour exposure was found to be about 150 µg/ml. Duration of treatment also influenced the ability of SuperFect to remove PrP^{Sc} from ScN2a cells: exposure to 150 µg/ml SuperFect for 10 min did not affect PrP^{Sc} levels, whereas 7.5 µg/ml SuperFect eliminated all detectable PrP^{Sc} with a $t_{1/2}$ = 8 h.

SuperFect is a mixture of branched polyamines derived from heat-induced degradation of a
25 PAMAM dendrimer (Tang, M.X., Redemann, C.T. & Szoka, F.C.J. In vitro gene delivery by degraded polyamidoamine dendrimers. *Bioconjug. Chem.* **7**, 703-714 (1996)). Knowing this structure the ability of several other branched and unbranched polymers to eliminate PrP^{Sc} from ScN2a cells (Table 1). The branched polymers investigated include various preparations of PEI, as well as intact PAMAM and PPI dendrimers. Dendrimers are manufactured by a repetitive divergent
30 growth technique, allowing the synthesis of successive, well-defined "generations" of homodisperse structures (Figure 1). The potency of both PAMAM and PPI dendrimers in eliminating PrP^{Sc} from ScN2a cells increased as the generation level increased. The most potent compounds with respect to eliminating PrP^{Sc} were PAMAM generation 4.0 and PPI generation 4.0, whereas PAMAM

generation 1.0 showed very little ability to eliminate PrP^{Sc} (Table 1). Similarly, a high MW fraction of PEI was more potent than low MW PEI.

From the foregoing data, it is clear that for all three branched polyamines tested, increasing molecular size corresponded to an increased potency for eliminating PrP^{Sc}. To determine whether this trend was directly attributable to increased surface density of amino groups on the larger molecules, PAMAM-OH generation 4.0 was tested. This is a dendrimer that resembles PAMAM generation 4.0 except that hydroxyls replace amino groups on its surface. Unlike PAMAM generation 4.0, PAMAM-OH generation 4.0 did not cause a reduction of PrP^{Sc} levels even at the highest concentration tested (10 mg/ml), establishing that the amino groups are required for the elimination of PrP^{Sc} by PAMAM (Table 1).

In an effort to assess the contribution of the branched architecture to the clearing ability of polyamines for PrP^{Sc}, the linear molecules poly-(L)lysine and linear PEI were also tested. Both of these linear compounds were less potent than a preparation of branched PEI with similar average molecular weight (Table 1), establishing that a branched molecular architecture optimizes the ability of polyamines to eliminate PrP^{Sc}, presumably because the branched structures achieve a higher density of surface amino groups.

Kinetics of PrP^{Sc} elimination by polyamines.

The preceding results demonstrate the potent ability of branched polyamines to clear PrP^{Sc} from ScN2a cells within a few hours of treatment. The utility of these compounds to act as therapeutics for treatment of prion disease was tested by determining whether they were cytotoxic for ScN2a cells, using as criteria cell growth, morphology, and viability as measured by trypan blue staining. None of the compounds was cytotoxic to ScN2a cells after exposure for one week at concentrations up to 7.5 μ g/ml. To determine whether branched polyamines can cure ScN2a cells of scrapie infection without affecting cell viability, the kinetics of prion clearance was examined in the presence of a non-cytotoxic concentration (7.5 μ g/ml) of three different branched polyamines. ScN2a cells were exposed to SuperFect, PEI, or PAMAM generation 4.0 for varying periods of time. The kinetics of PrP^{Sc} elimination were assessed by Western blotting. All three compounds caused a substantial reduction in PrP^{Sc} levels after 8-16 h of treatment, and of the three compounds, PEI appeared to remove PrP^{Sc} most quickly, with a $t_{1/2}$ = 4 h.

Curing neuroblastoma cells of scrapie infection.

The above results show that it is possible to reverse the accumulation of PrP^{Sc} in ScN2a cells under non-cytotoxic conditions. It was also found that extended exposure to even lower levels

of the branched polyamines (1.5 $\mu\text{g/ml}$) was sufficient to eliminate PrP^{Sc} . Based on these findings, this protocol was used to determine whether the severe reduction in PrP^{Sc} levels following exposure to branched polyamines would persist after removal of the compounds. Following the exposure of ScN2a cells to a 1.5 $\mu\text{g/ml}$ SuperFect for 1 week, PrP^{Sc} was reduced to <1% of the baseline level, but then increased back to ~5% of the baseline level after 3 additional weeks in culture in the absence of polyamine. In contrast, following exposure to 1.5 $\mu\text{g/ml}$ of either PEI or PAMAM generation 4.0 for 1 week, PrP^{Sc} was completely eliminated and did not return even after 3 weeks in culture without polyamines. A more intensive course of treatment with 1.8 $\mu\text{g/ml}$ SuperFect for 9 d also cured ScN2a cells of scrapie infection fully, manifested by the absence of PrP^{Sc} 1 month after removal of SuperFect.

Evidence for polyamines acting within an acidic compartment.

The above results showed the potent activity of branched polyamines in rapidly clearing scrapie prions from cultured ScN2a cells. Based on these results the mechanism by which these compounds act was investigated. All of the compounds which effect removal of PrP^{Sc} from ScN2a cells are known to traffic through endosomes (Boussif, O., *et al.* A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethyleneimine. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7297-7301 (1995); Haensler, J. & Szoka, F.C.J. Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjug. Chem.* **4**, 372-379 (1993)). Since PrP^{C} is converted into PrP^{Sc} in caveolae-like domains (CLDs) or rafts (Gorodinsky, A. & Harris, D.A. Glycolipid-anchored proteins in neuroblastoma cells form detergent-resistant complexes without caveolin. *J. Cell Biol.* **129**, 619-627 (1995); Taraboulos, A., *et al.* Cholesterol depletion and modification of COOH-terminal targeting sequence of the prion protein inhibits formation of the scrapie isoform. *J. Cell Biol.* **129**, 121-132 (1995); Vey, M., *et al.* Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. *Proc. Natl. Acad. Sci. USA* **93**, 14945-14949 (1996); Kaneko, K., *et al.* COOH-terminal sequence of the cellular prion protein directs subcellular trafficking and controls conversion into the scrapie isoform. *Proc. Natl. Acad. Sci. USA* **94**, 2333-2338 (1997)) and is then internalized through the endocytic pathway (Caughey, B., Raymond, G.J., Ernst, D. & Race, R.E. N-terminal truncation of the scrapie-associated form of PrP by lysosomal protease(s): implications regarding the site of conversion of PrP to the protease-resistant state. *J. Virol.* **65**, 6597-6603 (1991); Borchelt, D.R., Taraboulos, A. & Prusiner, S.B. Evidence for synthesis of scrapie prion proteins in the endocytic pathway. *J. Biol. Chem.* **267**, 16188-16199 (1992)), it was deduced that polyamines act upon PrP^{Sc} in endosomes or lysosomes. This deduction was investigated by determining the effect of pretreatment with the

lysosomotropic agents chloroquine and NH_4Cl on the ability of polyamines to eliminate PrP^{Sc} . These lysosomotropic agents alkalinize endosomes and have no effect on PrP^{Sc} levels when administered to ScN2a cells (Taraboulos, A., Raeber, A.J., Borchelt, D.R., Serban, D. & Prusiner, S.B. Synthesis and trafficking of prion proteins in cultured cells. *Mol. Biol. Cell* **3**, 851-863 (1992)). Experimental results obtained shows that 100 μM chloroquine, but not 30 μM NH_4Cl , blocked the ability of PEI to eliminate PrP^{Sc} . Similar results were obtained with SuperFect and PAMAM, generation 4.0. Although the failure of NH_4Cl to affect PrP^{Sc} levels is not easily explained, the ability of chloroquine to attenuate the ability of branched polyamines to remove PrP^{Sc} is consistent with the notion that these agents act in endosomes or lysosomes.

ORGAN HOMOGENATE ASSAY

The above results with cell cultures prompted investigating the possibility that in an acidic environment branched polyamines, either by indirectly interacting with PrP^{Sc} or with another cellular component, could cause PrP^{Sc} to become susceptible to hydrolases present in the endosome/lysosome. An *in vitro* degradation assay was developed to evaluate the effect of pH on the ability of polyamines to render PrP^{Sc} sensitive to protease. Crude homogenates of scrapie-infected mouse brain were exposed to a broad range of pH values in the presence or absence of SuperFect and then treated with proteinase K prior to Western blotting. Whereas PrP^{Sc} remained resistant to protease hydrolysis throughout the pH range (3.6-9.6) in the absence of Superfect, addition of the branched polyamine at pH 4.0 or below caused PrP^{Sc} to become almost completely degraded by protease.

Polyamine addition showed a dramatic effect on clearance *in vitro* which was optimized at pH 4 or less. These results show that polyamines act on PrP^{Sc} in an acidic compartment. To establish that the *in vitro* degradation assay is a valid approximation of the mechanism by which branched polyamines enhance the clearance of PrP^{Sc} from cultured cells, a structure activity analysis was performed with several of the compounds tested in culture cells. An excellent correlation was found between the clearance of PrP^{Sc} in cultured ScN2a cells (Table 1) and the ability to render PrP^{Sc} susceptible to protease at acidic pH *in vitro*. Notably, PAMAM-OH generation 4.0 failed to render PrP^{Sc} susceptible to protease, whereas PAMAM generation 4.0 and PPI, generation 4.0 exhibited an even stronger activity than Superfect *in vitro*, as expected from their observed potency in cultured ScN2a cells (Table 1).

MECHANISM OF ACTION

The results discussed here show that certain branched polyamines cause the rapid elimination of PrP^{Sc} from ScN2a cells in a dose- and time-dependent manner. These compounds demonstrate a potent ability to remove prions from cultured cells at concentrations that are completely non-cytotoxic. The cells may be maintained indefinitely in culture in the presence of therapeutic levels of branched polyamines. Furthermore, when ScN2a cells were exposed to these compounds for ~ 1 week, PrP^{Sc} was reduced to undetectable levels and remained so for at least one month after removal of the polyamine.

Clarification of the exact mechanism of PrP^{Sc} elimination by branched polyamines is an important objective. Although a number of possible scenarios exist, several possibilities may be excluded already. One possibility that was eliminated was that polyamines act by induction of chaperones such as heat shock proteins that mediate prion protein refolding because the above results show that it was possible to reproduce the phenomenon *in vitro*. Furthermore polyamines seem to offer advantages over other putative therapeutics that would seek to promote refolding: at very high concentrations, dimethyl sulfoxide (DMSO) and glycerol act as direct "chemical chaperones" and inhibit the formation of new PrP^{Sc} (Tatzelt, J., Prusiner, S.B. & Welch, W.J. Chemical chaperones interfere with the formation of scrapie prion protein. *EMBO J.* **15**, 6363-6373 (1996)), but these compounds cannot reduce pre-existing PrP^{Sc} levels. Furthermore, polyamines inhibit PrP^{Sc} formation at much lower concentrations than these agents. The ability of polyamines to effect the rapid clearance of PrP^{Sc} also contrasts with the activity of other potential prion therapeutics. Sulfated polyanions may inhibit PrP^{Sc} accumulation in ScN2a cells by directly binding to PrP^C (Gabizon, R., Meiner, Z., Halimi, M. & Ben-Sasson, S.A. Heparin-like molecules bind differentially to prion-proteins and change their intracellular metabolic fate. *J. Cell. Physiol.* **157**, (1993); Caughey, B., Brown, K., Raymond, G.J., Katzeinstein, G.E. & Thresher, W. Binding of the protease-sensitive form of PrP (prion protein) to sulfated glycosaminoglycan and Congo red. *J. Virol.* **68**, 2135-2141 (1994)), but because branched polyamines are able to clear pre-existing PrP^{Sc}, their mechanism of action cannot simply involve binding to PrP^C and inhibiting *de novo* synthesis.

Another possible mechanism which can be excluded is endosomal rupture. The branched polyamines which were effective in clearing PrP^{Sc} from ScN2a cells in our experiments, PEI, SuperFect and PAMAM, are also potent lysosomotropic, osmotic agents which can swell in acidic environments and rupture endosomes (Boussif, O., *et al.* A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethyleneimine. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7297-7301 (1995); Haensler, J. & Szoka, F.C.J. Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjug. Chem.* **4**, 372-379 (1993)). This might

- suggest that branched polyamines clear PrP^{Sc} from ScN2a cells by rupturing endosomes and exposing PrP^{Sc} to cytosolic degradation processes. However, it is known that the lysosomotropic, endosome-rupturing agents NH₄Cl, chloroquine, and monensin do not interfere with the formation of PrP^{Sc} in ScN2a cells (Taraboulos, A., Raeber, A.J., Borchelt, D.R., Serban, D. & Prusiner, S.B. 5 Synthesis and trafficking of prion proteins in cultured cells. *Mol. Biol. Cell* **3**, 851-863 (1992)). Furthermore, the results also show that chloroquine interferes with the ability of branched polyamines to clear PrP^{Sc} and that polyamines can clear PrP^{Sc} *in vitro* at acidic pH in the absence of cell membranes. Together, these observations rule out endosome rupture as the mechanism by which branched polyamines remove PrP^{Sc}.
- 10 Without committing to any particular mechanism of action it appears likely that branched polyamines require the acidic environment of intact endosomes or lysosomes to destroy PrP^{Sc}. The structure-activity profile of polymers tested reveals that the most active compounds possess densely packed, regularly-spaced amino groups, suggesting that these compounds may bind to a ligand which has periodically-spaced negative charges. Several scenarios remain possible. (1) Branched 15 polyamines may bind directly to PrP^{Sc} arranged as an amyloid with exposed negatively-charged moieties and induce a conformational change under acidic conditions. (2) Treatment of PrP 27-30 with acid decreases turbidity and increases α -helical content, suggesting that such conditions might dissociate PrP^{Sc} into monomers (Safar, J., Roller, P.P., Gajdusek, D.C. & Gibbs, C.J., Jr. Scrapie amyloid (prion) protein has the conformational characteristics of an aggregated molten globule 20 folding intermediate). It is therefore possible that polyamines bind to an equilibrium unfolding intermediate of PrP^{Sc} present under acidic conditions. (3) Alternatively, polyamines might sequester a cryptic, negatively charged component bound to PrP^{Sc} that is essential for protease resistance, but which is only released when PrP^{Sc} undergoes an acid-induced conformational change. Such a component might act as a chaperone for PrP^{Sc} inside endosomes or lysosomes. (4) Finally, another 25 possibility is that polyamines activate an endosomal or lysosomal factor which can induce a conformational change in PrP^{Sc}. Clearly, more work will be required to determine the precise mechanism by which branched polyamines destroy PrP^{Sc}.

GENERAL APPLICABILITY OF ASSAY

- 30 The *in vitro* assay described here is generally applicable in the search for compounds that effectively clear conformationally altered proteins present in food thereby preventing a number of degenerative diseases, where the accumulation of proteins seems to mediate the pathogenesis of these illnesses. By simulating lysosomes, where proteases hydrolyze proteins under acidic conditions, the

in vitro brain homogenate assay is able to rapidly evaluate the efficacy of a variety of polyamines to induce degradation of PrP^{Sc}.

The *in vitro* assay which used scrapie infected brain homogenate to test for compounds which clear PrP^{Sc} could be modified to assay for compounds which would clear any conformationally altered protein. The assay is carried out by homogenizing the organ or tissue where the
5 conformationally altered protein is present in the highest concentration. The pH of the homogenate is then reduced to less than 5.0 and preferably 4.0 or less. For example pancreatic tissue can be homogenized to produce an assay to test for compounds which clear amylin which is associated with type II Diabetes. Homogenized kidney could be used to test for compounds which clear β_2 -
10 microglobulin and homogenized heart or vascular tissue used to test for compounds which clear atrial natriuretic factor. Those skilled in the art will recognize other organs and tissue types which can be homogenized to test for other compounds which clear other conformationally altered proteins.

Besides using the *in vitro* assay to screen for potential drugs, the compounds found via the assay such as branched polyamines provide a new tool for exploring the conversion of a protein to
15 conformationally altered protein, e.g. PrP^C into PrP^{Sc}. The mechanism by which branched polyamines render PrP^{Sc} susceptible to proteolysis, remains to be established. Whether the interaction of branched polyamines with PrP^{Sc} is reversible is unknown. In addition, we do not know whether branched polyamines are able to solubilize PrP^{Sc} without irreversibly denaturing the protein. Whatever the mechanism by which branched polyamines interact with PrP^{Sc}, it is likely to be
20 different from that found with chaotropes as well as denaturing detergents and solvents (Prusiner, S.B., Groth, D., Serban, A., Stahl, N. & Gabizon, R. Attempts to restore scrapie prion infectivity after exposure to protein denaturants. *Proc. Natl. Acad. Sci. USA* 90, 2793-2797 (1993)).

Using the assays described and disclosed here certain specific branched polyamines have been found which mediate the clearance of PrP^{Sc} from cultured cells under non-cytotoxic conditions.
25 These compounds offer the intriguing possibility of being added to a wide range of low pH food products to neutralize conformational altered proteins present. Since the compounds found act by stimulating normal cellular pathways of protein degradation to destroy PrP^{Sc}, this class of compounds would also likely be of value in the treatment of other degenerative and hereditary disorders where abnormally folded, wild-type or mutant proteins accumulate. Such an approach
30 may find merit in developing an effective therapeutics for one or more of the common, degenerative illnesses including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, frontotemporal dementia, adult onset diabetes mellitus and the amyloidoses (Beyreuther, K. & Masters, C.L. Serpents on the road to dementia and death. Accumulating evidence from several studies points to the normal function of presenilin 1 and suggests how the mutant protein contributes

to deposition of amyloid plaques in Alzheimer's disease. *Nature Medicine* **3**, 723-725 (1997); Masters, C.L. & Beyreuther, K. Alzheimer's disease. *BMJ* **316**, 446-448 (1998); Selkoe, D.J. The cell biology of beta-amyloid precursor protein and presenilin in Alzheimer's disease. *Trends in Cell Biol.* **8**, 447-453 (1998); Selkoe, D.J. Translating cell biology into therapeutic advances in

5 Alzheimer's disease. *Nature* **399**, A23-31 (1999); Wong, P.C., *et al.* An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. *Neuron* **14**, 1105-1116 (1995); Spillantini, M.G., Crowther, R.A., Jakes, R., Hasegawa, M. & Goedert, M. α -Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. *Proc. Natl. Acad. Sci. USA* **95**, 6469-6473

10 (1998); Hutton, M., *et al.* Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. *Nature* **393**, 702-705 (1998); Stone, M.J. Amyloidosis: a final common pathway for protein deposition in tissues. *Blood* **75**, 531-545 (1990)). Whether branched polyamines might also prove efficacious in a variety of inherited disorders where the accumulation of abnormal proteins is a hallmark of the illness remains to be established; these genetic maladies

15 include heritable forms of prion disease, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, frontotemporal dementia, Pick's disease and amyloidosis, as well as the triplet repeat diseases including Huntington's disease, spinal cerebellar ataxias and myotonic dystrophy (Fu, Y.-H., *et al.* An unstable triplet repeat in a gene related to myotonic muscular dystrophy. *Science* **255**, 1256-1259 (1992); Group, T.H.s.D.C.R. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* **72**, 971-983 (1993)).

20 Compounds identified via assays of the invention such as branched polyamines will find utility in preventing or delaying the onset of these genetic diseases where carriers can often be identified decades in advance of detectable neurologic or systemic dysfunction.

The invention is based on the discovery that several dendritic polycations, including the

25 starburst dendrimers Superfect™ (QIAGEN®, Valencia, CA), polyamidoamide (PAMAM), and the hyperbranched polycation polyethyleneimine (PEI), were surprisingly found to eliminate PrP^{Sc} from cultured scrapie-infected neuroblastoma cells. These highly-branched, polycationic compounds provide a novel class of therapeutic agents to combat prion diseases and other degenerative disease including the amyloidoses. The removal of PrP^{Sc} is dependent on both the concentration of dendritic

30 polymer and length of exposure. Dendritic polymers were able to clear PrP^{Sc} at concentrations which were not cytotoxic. Repeated exposures to heat-degraded starburst PAMAM dendrimer or PEI caused a dramatic reduction in PrP^{Sc} levels which persisted for a month even after removal of the compound. Dendritic polycations did not appear to destroy purified PrP^{Sc} *in vitro*, and therefore may act through a generalized mechanism. Dendritic polycations represent a class of compounds which

can be used as therapeutic agents in prion diseases and other disorders involving insoluble protein deposits, such as the amyloidoses.

EXAMPLES

5 The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (*e.g.* amounts, temperature, etc.) but some
10 experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

METHODS AND MATERIALS

15 **Chemicals.** High molecular weight PEI was purchased from Fluka. DOTAP cationic lipid was purchased from Boehringer Mannheim and SuperFect transfection reagent was purchased from QIAGEN®. All other compounds were purchased from Sigma-Aldrich. All test compounds were dissolved in water at stock concentration of 3 mg/ml and filtered through a Millipore 0.22 m m filter.

20 **Cultured cells.** Stock cultures of ScN2a cells were maintained in MEM with 10% FBS, 10% Glutamax (Gibco BRL), 100 U penicillin, and 100 mg/ml streptomycin (supplemented DME). Immediately prior to addition of test compounds, the dishes were washed twice with fresh supplemented DME media. After exposure to test compounds, dishes were drained of media and cells were harvested by lysis in 0.25-1 ml 20 mM Tris pH 8.0 containing 100 mM NaCl, 0.5%
25 NP-40, and 0.5% sodium deoxycholate to obtain a total protein concentration of 1 mg/ml measured by the BCA assay. Nuclei were removed from the lysate by centrifugation at 2000 rpm for 5 min. For samples not treated with proteinase K, 40 μ l of whole lysate (representing 40 μ g total protein) was mixed with an equal volume of 2x SDS reducing sample buffer. For proteinase K digestion, 20
30 μ g/ml proteinase K (Boehringer Mannheim) (total protein:enzyme ratio = 50:1) was added, and the sample was incubated for 1 h at 37°C. Proteolytic digestion was terminated by the addition of Pefabloc to a final concentration of 5 mM. One ml samples were centrifuged at 100,000 x g for 1 h at 4°C, the supernatants were discarded, and the pellets were resuspended in 80 μ l of reducing SDS sample buffer for SDS-PAGE.

Brain homogenates. Brain homogenates from RML scrapie-affected CD-1 mice (10% (w/v) in sterile water) were prepared by repeated extrusion through syringe needles of successively smaller size, from 18 to 22 gauge. Nuclei and debris were removed by centrifugation at 1000 x g for 5 min. The bicinchoninic acid (BCA) protein assay (Pierce) was used to determine protein concentration. Homogenates were adjusted to 1 mg/ml protein in 1% NP-40. For reactions, 0.5 ml homogenate was incubated with 25 ml 1.0 M buffer (sodium acetate for pH 3-6 and Tris acetate for pH 7-10) plus or minus 10 ml of polyamine stock solution (3 mg/ml) for 2 h at 37°C with constant shaking. The final pH value of each sample was measured directly with a calibrated pH electrode (Radiometer Copenhagen). Following incubation, each sample was neutralized with an equal volume 0.2 M HEPES pH 7.5 containing 0.3 M NaCl and 4% Sarkosyl. Proteinase K was added to achieve a final concentration of 20 µg/ml, and samples were incubated for 1 h at 37°C. Proteolytic digestion was terminated by the addition of Pefabloc to a final concentration of 5 µM. Ten µl of digested brain homogenate was mixed with equal volume 2x SDS sample buffer and analyzed by SDS-PAGE followed by Western blotting.

Western blotting. Following electrophoresis, Western blotting was performed as previously described (Scott, M., *et al.* Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques. *Cell* **59**, 847-857 (1989)). Samples were boiled for 5 min and cleared by centrifugation for 1 min at 14,000 rpm in a Beckman ultrafuge. SDS-PAGE was carried out in 1.5 mm, 12% polyacrylamide gels (Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T-4. *Nature* **227**, 680-685 (1970)). Membranes were blocked with 5% non-fat milk protein in PBST (calcium- and magnesium-free PBS plus 0.1% Tween 20) for 1 h at room temperature. Blocked membranes were incubated with primary RO73 polyclonal antibody (to detect MoPrP) (Serban, D., Taraboulos, A., DeArmond, S.J. & Prusiner, S.B. Rapid detection of Creutzfeldt-Jakob disease and scrapie prion proteins. *Neurology* **40**, 110-117 (1990)) or 3F4 monoclonal antibody (to detect MHM2 PrP) (Kascsak, R.J., *et al.* Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. *J. Virol.* **61**, 3688-3693 (1987)) at 1:5000 dilution in PBST overnight at 4°C. Following incubation with primary antibody, membranes were washed 3 x 10 min in PBST, incubated with horseradish peroxidase-labeled secondary antibody (Amersham Life Sciences) diluted 1:5000 in PBST for 30 to 60 min at 4°C and washed again for 3 x 10 min in PBST. After chemiluminescent development with ECL reagent (Amersham) for 1 min, blots were sealed in plastic covers and exposed to ECL Hypermax film (Amersham). Films were processed automatically in a Konica film processor.

EXAMPLE 1A

Branched polyamines inhibit formation of nascent PrP^{Sc} and induce clearance of pre-existing PrP^{Sc}

Western blots were probed with 3F4 monoclonal antibody which recognizes newly expressed
5 MHM2 PrP. ScN2a cells were exposed to SuperFect for 3 h and harvested 3 d after removal of SuperFect. Gels were run on both undigested, control sample and a sample subjected to limited proteolysis. The samples were run in separate lanes 1-6 with a control and limited proteolysis sample for each of the 6 lanes as follows: Lane 1: DOTAP-mediated transfection. Lane 2: 30 $\mu\text{g/ml}$ SuperFect, 5 μg pSPOX MHM2. Lane 3: 75 $\mu\text{g/ml}$ SuperFect, 5 μg pSPOX MHM2. Lane 4: 150
10 $\mu\text{g/ml}$ SuperFect, 5 μg pSOX MHM2. Lane 5: 150 $\mu\text{g/ml}$ SuperFect, 10 μg pSPOX MHM2. Lane 6: No addition of either transfection reagent or DNA. Forty μl of undigested brain homogenate was used in these studies while those samples subjected to limited digestion with proteinase K were concentrated 25-fold prior to SDS-PAGE. One ml of the digest were centrifuged at 100,000 $\times g$ for 1 h at 4°C and the pellets suspended in 80 μl of SDS sample buffer prior to SDS-PAGE followed by
15 Western blotting. Apparent molecular weights based on migration of protein standards are 34.2, 28.3, and 19.9 kDa.

All of the control lanes 1-6 show multiple bands as expected. However, of the samples subjected to limited proteolytic only lane 1 shows bands. Unexpectedly, all of the partially digested sample lanes 2-5 show no bands and as expected no bands in the partially digested lane 6. These
20 results show the effect of using SuperFect in clearing PrP^{Sc}.

EXAMPLE 1B

The blot described above was stripped of antibody, exposed to labeled R073 and redeveloped. The antibody 3F4 used in Example 1 binds to PrP^C but not to PrP^{Sc}. However, R073
25 binds to PrP^{Sc} and PrP^C. Lanes 1, 2 and 3 show decreasing amounts of PrP^{Sc} and lanes 4 and 5 show no detectable PrP^{Sc}.

EXAMPLE 2A

Gels were run on undigested controls 1-4 and as above, samples subjected to limited
30 proteolysis. The lanes were as follows: Lane 1: No SuperFect. Lane 2: 30 $\mu\text{g/ml}$ SuperFect. Lane 3: 75 $\mu\text{g/ml}$ SuperFect. Lane 4: 150 $\mu\text{g/ml}$ SuperFect. ScN2a cells were exposed to SuperFect for 3 h and harvested 3 d after removal of SuperFect. Apparent molecular weights based on migration of protein standards are 33.9, 28.8, and 20.5 kDa. In that each sample was tested after the same

time period the results show the dose-dependent effect of SuperFect on PrP^{Sc} removal. Lanes 1, 2 and 3 show decreasing amounts of PrP^{Sc} and lane 4 shows no detectable PrP^{Sc}.

EXAMPLE 2B

5 To determine the time-dependent effect of SuperFect three different panels with four lanes each were prepared and run as follows: ScN2a cells were exposed to 7.5 μ g/ml: SuperFect (lanes 1-4), PEI (average molecular weight ~60,000)(lanes 5-8), or PAMAM, generation 4.0 (lanes 9-12). Time of exposure times for each polyamine: 0 hours (lanes 1, 5, and 9), 4 hours (lanes 2, 6, and 10), 8 hours (lanes 3, 7, and 11), 16 hours (lanes 4, 8, and 12). All samples were subjected to limited
10 proteolysis to measure PrP^{Sc}. Apparent molecular weights based on migration of protein standards are 38, 26, and 15 kDa. Lanes of each of the three panels show decreasing amounts of PrP^{Sc}.

EXAMPLE 3

In this example four panels A,B, C and D were created with panels having three double
15 (control and test) lanes each. ScN2a cells were exposed to 1.5 μ g/ml: (A) SuperFect, (B) PEI (average molecular weight ~60,000), (C) PAMAM, generation 4.0, or (D) no addition. Cells were harvested: Lane 1, before addition; Lane 2, immediately following 1 week continuous exposure to test compounds; and Lane 3, three weeks after removal of test compounds. Minus (-) symbol denotes undigested, control sample and plus (+) symbol designates sample subjected to limited
20 proteolysis. Apparent molecular weights based on migration of protein standards are 33.9, 28.8, and 20.5 kDa. Test lanes 3 in panel A showed slight PrP^{Sc} after three weeks and test lanes 3 in panels B and C showed no detectable PrP^{Sc} whereas PrP^{Sc} was present in all lanes in panel D.

EXAMPLE 4A

25 Four separate gels were run to demonstrate the effect of adding chloroquine would have on PrP^{Sc} levels. The lanes 1 control and 3 where chloroquine was added show clear bands for PrP^{Sc} whereas lanes 2 and 4 with no chloroquine show barely detectable amounts of PrP^{Sc}. The four lanes were prepared as follows: ScN2a cells were treated Lane 1: Control media. Lane 2: 7.5 μ g/ml PEI (average molecular weight ~60,000). Lane 3: PEI plus 100 μ M chloroquine. Lane 4: PEI plus 30
30 μ M NH₄Cl. Chloroquine and NH₄Cl were added 1 h prior to addition of PEI. Cells were harvested 16 hours after addition of PEI. All samples shown were subjected to limited proteolysis to measure PrP^{Sc}. Apparent molecular weights based on migration of protein standards are 38, 26, and 15 kDa.

EXAMPLE 4B

Eight lanes with SuperFect (+SF) and eight lanes without SuperFect (-SF) were prepared. Lanes 1-8 of each group had an adjusted pH of 3.6, 4, 5, 6, 7, 8, 9 and 9.6. *In vitro* mixture of crude mouse brain homogenates with SuperFect under a range of pH conditions was performed as described in methods (measured final pH of each sample denoted above the lanes). Addition of 60 $\mu\text{g/ml}$ SuperFect denoted as "+SF" and control with no addition as "-SF". All samples shown were subjected to limited proteolysis to measure PrP^{Sc} . Apparent molecular weights based on migration of protein standards are 30 and 27 kDa. All lanes of the -SF group showed PrP^{Sc} present. Lanes 3-8 of the +SF group showed PrP^{Sc} . However, lanes 1 and 2 with respective pH levels of 3.6 and 4.0 showed very slight detectable PrP^{Sc} . The results show that the ability of a blanching polycation such as SuperFect to clear PrP^{Sc} is pH dependent.

EXAMPLE 5

Sixteen different lanes were prepared as described. Lanes 1 and 2 were control lanes and each of lanes 3-16 contained a different compound as tested in Table 1. The test compounds were all polyamines. Thus, the results show removal of PrP^{Sc} from brain homogenate *in vitro* by various polyamines. Samples were incubated with polyamines at pH 3.6 and processed as described in Methods. Each polyamine was tested at 60 $\mu\text{g/ml}$ concentration. Lanes 1 and 2: control. Lane 3: poly-(L)lysine. Lane 4: PAMAM, generation 0.0. Lane 5: PAMAM, generation 1.0. Lane 6: PAMAM, generation 2.0. Lane 7: PAMAM, generation 3.0. Lane 8: PAMAM, generation 4.0. Lane 9: PAMAM-OH, generation 4.0. Lane 10: PPI, generation 2.0. Lane 11: PPI, generation 4.0. Lane 12: linear PEI. Lane 13: high MW PEI. Lane 14: low MW PEI. Lane 15: average MW PEI. Lane 16: SuperFect. All samples shown were subjected to limited proteolysis to measure PrP^{Sc} . Apparent molecular weights based on migration of protein standards are 30 and 27 kDa. **Table 1.** Removal of PrP^{Sc} by polymer compounds. IC_{50} = approximate concentration of polymer required to reduce PrP^{Sc} to 50% of control levels in ScN2a cells after exposure for 16 hours. All compounds were tested at 5 different concentrations. PrP^{Sc} levels were measured by densitometry of Western blot signals.

TABLE 1

(Note that Table 1 includes information on the characteristics of compounds used but that the list does not correspond directly to lanes 1-16)

5	Compound	Molecular Weight	Primary NH ₂ groups	IC ₅₀ (ng/ml)
	PAMAM generation 0.0	517	4	>10,000
	PAMAM generation 1.0	1,430	8	>10,000
10	PAMAM generation 2.0	3,256	16	2,000
	PAMAM generation 3.0	6,909	32	400
	PAMAM generation 4.0	14,215	64	80
	PAMAM-OH generation 4.0	14,279	0	>10,000
	PPI generation 2.0	773	8	2,000
15	PPI generation 4.0	3,514	32	80
	Low MW PEI	~25,000		2,000
	Average MW PEI	~60,000		400
	High MW PEI	~800,000		80
	Linear PEI	~60,000		2,000
20	poly-(L)lysine	~60,000	>500	10,000
	SuperFect			400

Lanes 7, 8, 11 and 13 showed the best results, i.e. best ability to clear PrP^{Sc} under these conditions. Specifically, PAMAM generation 4.0 in lane 8 showed the best ability to clear PrP^{Sc} under these conditions whereas PAMAM-OH generation 4.0 showed almost no detectable ability to clear PrP^{Sc} and was comparable to the control.

EXAMPLE 6

Transfection of PrP^{Sc} Expressing Cells with Dendrimer Compounds

Cells of neuronal origin expressing PrP^{Sc} were examined for the ability of compounds to suppress PrP^{Sc} formation.

Transfection Studies

Stock cultures of N2a and ScN2a cells were maintained in MEM with 10% FBS, 10% Glutamax (Gibco BRL), 100 U penicillin, and 100 µg/ml streptomycin. Cells from a single confluent 100 mm dish were trypsinized and split into 10 separate 60 mm dishes containing DME plus 10% FBS, 10% Glutamax, 100 U penicillin, and 100 µg/ml streptomycin (supplemented DME) one day prior to transfection. Immediately prior to transfection, the dishes were washed twice with 4 ml supplemented DME media and then drained.

For DOTAP-mediated transfection, 15 μ g pSPOX MHM2 was resuspended in 150 μ l sterile Hepes Buffered Saline (HBS) on the day of transfection. The DNA solution was then mixed with an equal volume of 333 μ g/ml DOTAP (Boehringer Mannheim) in HBS in Falcon 2059 tubes and incubated at room temperature for 10 minutes to allow formation of DNA/lipid complexes.

- 5 Supplemented DME (2.5 ml) was added to the mixture, and this was then pipetted onto drained cell monolayers. The following day, the medium containing DNA/lipid was removed and replaced with fresh supplemented DME. Cells were harvested three days later.

- For Superfect™-mediated transfections/exposures, Superfect™ with or without DNA was added to 1 ml supplemented DME in a Falcon 2059 tube to achieve the specific concentrations
10 needed for each experiment. This mixture was pipetted up and down twice and then onto drained cell monolayers. After exposure for the indicated times, the medium containing Superfect™ was removed and replaced with fresh supplemented DME. Cells were harvested at specified times after removal of Superfect™.

- Exposures to PPI (DAB-Am-8, Polypropylenimine octaamine Dendrimer, Generation 2.0
15 Aldrich 46,072-9), Intact PAMAM (Starburst (PAMAM)Dendrimer, Generation 4.
Aldrich 41,244-9), PEI (Sigma), poly-(L)lysine (Sigma), and poly-(D) lysine (Sigma) were performed as described above for Superfect™.

Isolation of Protein from Treated Cells

- 20 Cells were harvested by lysis in 1.2 ml of 20 mM Tris pH 8.0 containing 100 mM NaCl, 0.5% NP-40, and 0.5% sodium deoxycholate. Nuclei were removed from the lysate by centrifugation at 2000 rpm for 5 min. This lysate typically had a protein concentration of 0.5 mg/ml measured by the BCA assay. For samples not treated with proteinase K, 40 μ l of whole lysate (representing 20 μ g total protein) was mixed with 40 μ l of 2x SDS sample buffer. For proteinase K
25 digestion, 1 ml of lysate was incubated with 20 μ g/ml proteinase K (total protein:enzyme ratio = 25:1) for 1 hr at 37°C. Proteolytic digestion was terminated by the addition of 8 μ l of 0.5M PMSF in absolute ethanol. Samples were then centrifuged for 75 min in a Beckman TLA-45 rotor at 100,000 x g at 4°C. The pellet was resuspended by repeated pipetting in 80 μ l of 1X SDS sample
30 buffer. The entire sample (representing 0.5 mg total protein before digestion) was loaded for SDS-PAGE.

Western Blot Analysis

Immunoreactive PrP bands from the DOTAP-mediated transfection were detected before and after digestion with proteinase K with monoclonal antibody 3F4. The construct used to express PrP^{Sc} in the ScN2a cells is MHM2 a chimeric construct that differs from wild-type (wt) MoPrP at positions 108 and 111 (Scott et al., (1992) *Protein Sci.* 1:986-997). Substitution at these positions with the corresponding residues (109 and 112 respectively) from the Syrian hamster (SHa) PrP sequence creates an epitope for 3F4 (Kascsak et al., (1987) *J. Virol.* 61:3688-3693), which does not recognize endogenous wt MoPrP in ScN2a cells and hence facilitates specific detection of the transgene by Western blot.

Following electrophoresis, Western blotting was performed as previously described (Scott et al., (1989) *Cell* 59:847-857). Samples were boiled for 5 minutes and cleared by centrifugation for 1 minute at 14,000 rpm in a Beckman ultrafuge. SDS-PAGE was carried out in 1.5 mm, 12% polyacrylamide gels (Laemmli (1970) *Nature* 227:661-665). Membranes were blocked with 5% nonfat milk protein in PBST (calcium- and magnesium-free PBS plus 0.1% Tween 20) for 1 hour at room temperature. Blocked membranes were incubated with primary RO73 polyclonal or 3F4 monoclonal antibody at a 1:5000 dilution in PBST overnight at 4 °C.

Following incubation with primary antibody, membranes were washed 3 x 10 minutes in PBST, incubated with horseradish peroxidase-labeled secondary antibody (Amersham Life Sciences) diluted 1:5000 in PBST for 25 minutes at room temperature and washed again for 3x 10 minutes in PBST. After chemiluminescent development with ECL reagent (Amersham) for 1 minute, blots were sealed in plastic covers and exposed to ECL Hypermax film (Amersham). Films were processed automatically in a Konica film processor.

In contrast to DOTAP-transfected cells, ScN2a cells transfected with varying concentrations of SuperfectTM and DNA did not appear to contain protease-resistant MHM2. Close scrutiny revealed that, prior to protease digestion, SuperfectTM-transfected samples express MHM2 bands which are not seen in the background pattern of the control sample. These observations indicate that MHM2 PrP was successfully expressed using SuperfectTM transfection reagent, but conversion of MHM2 PrP^C to protease-resistant MHM2 PrP^{Sc} was inhibited by SuperfectTM.

To examine whether SuperfectTM had affected levels of preexisting PrP^{Sc} in ScN2a cells, the Western blot probed with 3F4 antibody was reprobed with polyclonal antibody RO73, which is able to recognize endogenous MoPrP. Remarkably, SuperfectTM caused the disappearance of preexisting MoPrP^{Sc} from ScN2a cells in a dose-dependent manner. After treatment with SuperfectTM, PrP^{Sc} could not be detected in the nuclear fraction, pellet, supernatant, or media. The concentration of SuperfectTM required to fully remove preexisting PrP^{Sc} with a three hour exposure was 300 µg/ml,

whereas 30 µg/ml was sufficient to interfere with the formation of new MHM2 PrP^{Sc} within the same time frame.

Length of exposure dramatically influenced the ability of Superfect™ to remove PrP^{Sc} from ScN2a cells. Whereas a 3 hour exposure to 150 µg/ml Superfect™ significantly lowered PrP^{Sc} levels in ScN2a cells, exposure for 10 min to the same dose of Superfect™ did not affect PrP^{Sc} levels. When ScN2a cells were exposed to 2 µg/ml Superfect™ continuously for 1 week, PrP^{Sc} disappeared completely.

The conditions tested did not appear to be toxic for the cells. Neither 150 µg/ml Superfect™ for 3 hrs nor 2 µg/ml Superfect™ continuously for 1 week caused any obvious changes in cell morphology, viability, or growth as judged by phase contrast microscopy.

EXAMPLE 7

Elimination of PrP^{Sc} by repeated exposures to Superfect™

The duration in the reduction in PrP^{Sc} levels after exposure to Superfect™ was examined, and it was shown that this reduction could persist for extended periods after removal of Superfect™. Following the exposure of ScN2a cells to a single dose of 150 µg/ml Superfect™ for 3 hrs, PrP^{Sc} levels remained low for one week, but returned to near baseline levels after 3 weeks in culture without Superfect™

In contrast, when ScN2a cells were exposed to 4 separate doses of Superfect™ over the course of 16 days, very little PrP^{Sc} could be detected 4 weeks after the final exposure to Superfect™. This result offers hope that prolonged exposure to Superfect™ may lead to long term cure of scrapie infection in cultured cells.

EXAMPLE 8

Superfect™ does not destroy PrP^{Sc} directly

The dendrimer Superfect™ was used to determine if it could exert a similar inhibitory effect on PrP^{Sc} in either crude brain homogenates or purified PrP 27-30 rods. Brain homogenates from normal and scrapie-affected Syrian hamsters (10% (w/v) in sterile PBS) were prepared by repeated extrusion through syringe needles of successively smaller size, from 18 to 22 gauge. Nuclei and debris were removed by centrifugation at 1000 x g for 10 min. The bicinchoninic acid (BCA) protein assay (Pierce) was used to determine protein concentration. Homogenates were adjusted to 10 mg/ml protein with PBS and 50 µl was added to 450 µl of lysis buffer containing 100 mM NaCl, 1 mM EDTA, 0.55% sodium deoxycholate, 0.55% Triton X-100, and 50 mM Tris-HCl pH 7.5. This mixture was then incubated with 0-300 µg/ml Superfect™ for 3

hrs at 37 °C and then centrifuged for 10 min at 14,000 rpm in a Beckman Ultrafuge. The pellet was resuspended in 450 µl lysis buffer without Superfect™. Proteinase K (Boehringer Mannheim) was added to achieve a final concentration of 20 µg/ml, and thus the ratio of total protein/enzyme was 50:1. Samples were incubated for 1 h at 37 °C. Proteolytic digestion was terminated by the addition of 8 µl of 0.5 M PMSF in ethanol. Samples were then centrifuged for 75 min in a Beckman TLA-45 rotor at 100,000 x g at 4 °C. Undigested samples (10 µl) were mixed with an equal volume of 2x SDS sample buffer. For digested samples, the pellet was resuspended by repeated pipetting in 100 µl 1x SDS sample buffer. Twenty µl (equivalent to 100 µg of total protein prior to proteinase K digestion) of each sample was loaded for SDS-PAGE.

PrP 27-30 rods were purified from scrapie-affected Syrian hamster brains and previously described (Prusiner et al., (1983) *Cell* 35:349-358). Purified rods (3.5 µg/ml) were incubated with or without 900 µg/ml Superfect™ in 100 µl supplemented DME. After 16 hrs at 37 °C, the suspension was centrifuged at 100,000 x g at 4 °C. The pellet was resuspended in 500 µl of buffer containing 1 mg/ml BSA, 100 mM NaCl, 1 mM EDTA, 0.55% sodium deoxycholate, 0.55% Triton X-100, and 50 mM Tris-HCl pH 7.5. Proteinase K was added to achieve a final concentration of 20 µg/ml. Samples were incubated for 1 h at 37 °C. Proteolytic digestion was terminated by the addition of 8 µl of 0.5 M Pefabloc (Boehringer Mannheim). Samples were then centrifuged for 75 min at 100,000 x g at 4 °C. Undigested samples (50 µl) were mixed with an equal volume of 2x SDS sample buffer. For digested samples, the pellet was resuspended by repeated pipetting in 100 µl 1x SDS sample buffer. Forty µl of each sample was loaded for SDS-PAGE.

When Superfect™ was mixed with either crude homogenates of scrapie-affected Syrian hamsters or with purified Syrian hamster PrP 27-30, there was no significant change in the level of proteinase K-resistant PrP^{Sc}. These results suggest that the removal of PrP^{Sc} from ScN2a cells by Superfect™ depends on the presence of intact cellular machinery.

EXAMPLE 9

Clearance of PrP^{Sc} levels by other dendritic polycations

The Superfect™ compound is a high molecular weight component of heat-degraded PAMAM Starburst dendrimers, which is a cationic, highly-branched, monodisperse polymers (Tang et al., (1996) *Bioconjugate Chem.* 7:703-714). To identify other potentially useful anti-prion therapeutic agents, we screened three other dendritic polycations and two linear cationic polymers for their ability to clear PrP^{Sc} from ScN2a cells. Among the dendritic macromolecules tested, polyetheleneimine (PEI) was the most potent, removing the majority of PrP^{Sc} from ScN2a cells after 3 hrs when used at a concentration of 10 µg/ml. Intact PAMAM displayed a potency comparable to

Superfect™, removing approximately half of the detectable PrP^{Sc} when used at a concentration of 50 µg/ml. In contrast, the dendrimer polypropyleneimine (PPI), poly-(L)lysine, and the linear polycation poly-(D)lysine failed to reduce PrP^{Sc} levels at concentrations between 10-50 µg/ml. These results demonstrate that a branched polymeric architecture is required to clear PrP^{Sc}.

- 5 Furthermore, exposure of ScN2a cells to either PEI or intact PAMAM for one week at a concentration of 1.5 µg/ml completely removes PrP^{Sc}, effectively curing the cells of scrapie infection.

10 While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

CLAIMS

That which is claimed is:

1. A method of sterilizing an object, comprising the steps of:
5 contacting the object with a composition of a branched polycation at a pH of 5.0 or less; and
allowing the composition to remain in contact with the object for a period of time sufficient
to render a conformationally altered protein non-infectious.
2. The method of claim 1, further comprising:
10 removing the composition from the object.
3. The method of claim 1, wherein the branched polycation is a polycationic dendrimer
selected from the group consisting of polypropylene imine, polyethyleneimine (PEI)
poly(4'-aza-4'-methylheptamethylene D-glucaramide), polyamidoamines and variants or fragments
15 thereof.
4. The method of claim 1, wherein the object is a cell culture.
5. The method of claim 1, wherein the object is a bovine product.
20
6. A composition, comprising:
water in an amount of from 1% to 99.99% by weight;
polycationic dendrimer in an amount of 0.001% to 10% by weight.
25
7. The composition of claim 6, wherein the composition is a pharmaceutical
composition comprising a therapeutically effective amount of a branched polycation and
a pharmaceutically acceptable excipient.
8. The composition of claim 6, further comprising a second compound selected from
30 the group consisting of: a detergent, an antibacterial compound, an antiviral compound, and an
antifungal compound.

9. A capsule for oral administration of a compound, comprising:
gelatin extracted from a cow; and
a branched polycation.

5 10. A cell culture, comprising:
cells genetically engineering to produce a pharmaceutical;
cell nutrients; and
a branched polycation.

10

1/1

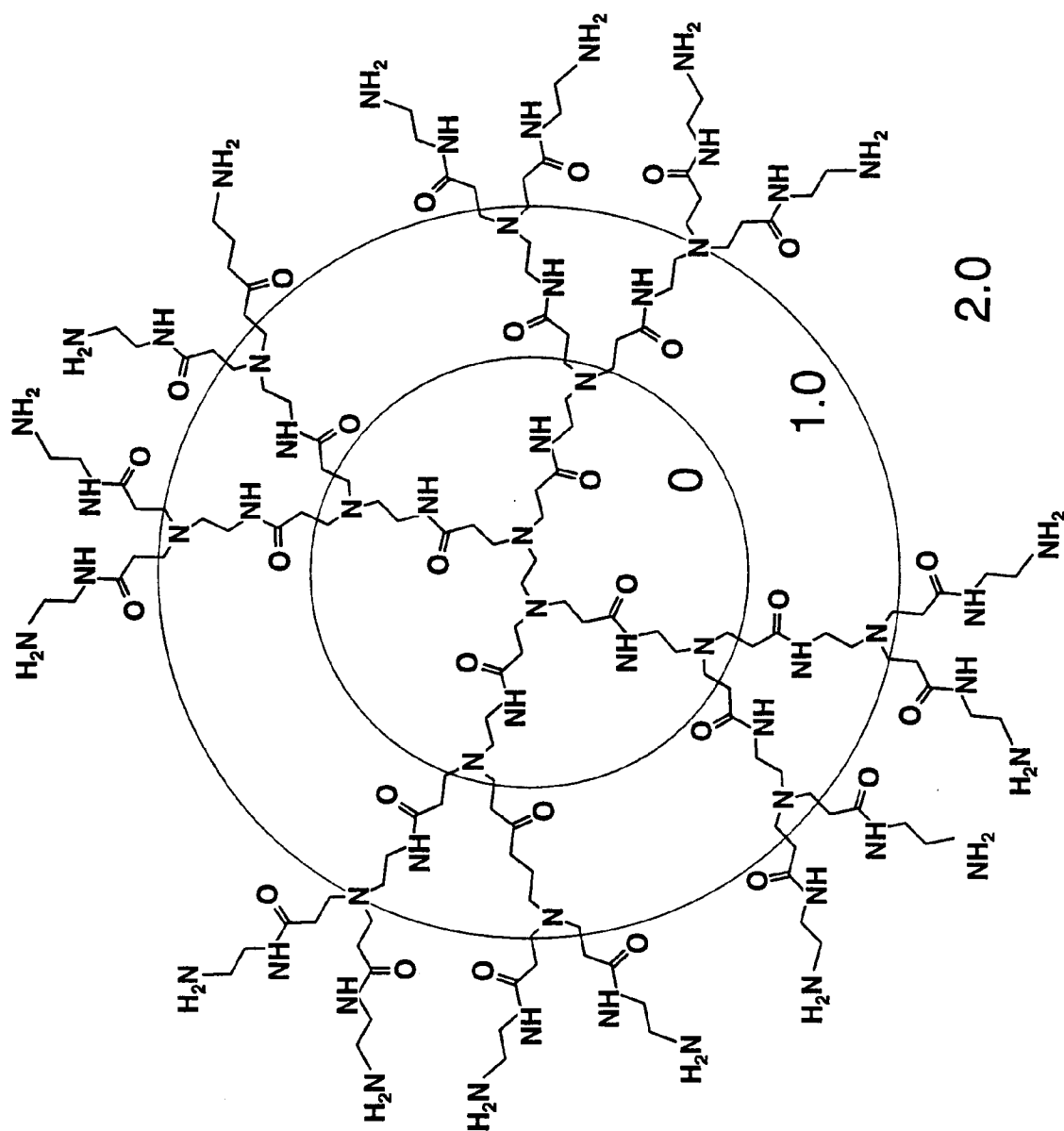


FIGURE 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/14353

A. CLASSIFICATION OF SUBJECT MATTER		
IPC(7) : A61K 31/74 US CL : 424/78.17		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
U.S. : 424/78.17 ,456		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	US 5,919,442 A (YIN et al) 06 July 1999, see Fig. 32, & Cols 10, 18, 19, 29, & 33, & example 17.	1-3, 6-8, 10
X	US 5,499,979 A (WONG et al) 19 March 1996, Cols 5, 7, 8.	9
X	US 5,834,020 (MARGERUM et al) 10 November 1998, see Cols 29, 30	1-4, 6-8
X	US 4,587,329 A (TOMALIA et al) 06 May 1986, see Cols 15-18.	6, 7
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art *&* document member of the same patent family	
A document defining the general state of the art which is not considered to be of particular relevance		
E earlier document published on or after the international filing date		
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search	Date of mailing of the international search report	
23 JUNE 2000	24 JUL 2000	
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NEIL LEVY <i>Neil Levy for</i> Telephone No. (703) 308-1235	